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Deoxycytidine Kinase from Human Leukemic Spleen: Preparation and Characterization of the Homogeneous Enzyme†

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Received July 10, 1987; Revised Manuscript Received February 2, 1988

ABSTRACT: Deoxycytidine kinase from human leukemic spleen has been purified 6000-fold to apparent homogeneity with an overall yield of 10%. The purification was achieved by using DEAE chromatography, hydroxylapatite chromatography, and affinity chromatography on dTTP–Sepharose. Only one form of deoxycytidine kinase activity was found during all the chromatographic procedures. The subunit molecular mass, as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, was 30 kilodaltons. The pure enzyme phosphorylates deoxycytidine, deoxyadenosine, and deoxyguanosine, demonstrating for the first time that the same enzyme molecule has the capacity to use these three nucleosides as substrates. The apparent molecular weight of the active enzyme, determined by gel filtration and glycerol gradient centrifugation, was 60000. Thus, the active form of human deoxycytidine kinase is a dimer. The kinetic behavior of pure human deoxycytidine kinase was studied in detail with regard to four different phosphate acceptors and two different phosphate donors. The apparent K_m values were 1, 20, 150, and 120 μM for deoxycytidine, arabinosylcytosine, deoxyguanosine, and deoxyadenosine, respectively. The V_{max} values were 5-fold higher for the purine nucleosides as compared to the pyrimidine substrates. We observe competitive inhibition of the phosphorylation of one substrate by the presence of either of the three other substrates, but the apparent K_i values differed greatly from the corresponding K_m values, suggesting the existence of allosteric effects. The double-reciprocal plots for ATP·$MgCl_2$ as phosphate donor were convex, indicating negative cooperative effects. In contrast, plots with varying dTTP·$MgCl_2$ concentration as phosphate donor were linear with an apparent K_m of 2 μM. The enzyme activity was strongly inhibited by dCTP, in a noncompetitive way with deoxycytidine and in a competitive way with ATP·$MgCl_2$.

Deoxycytidine kinase (dCyd kinase) (NTP:deoxycytidine 5′-phosphotransferase, EC 2.7.1.74) catalyzes the phosphorylation of deoxycytidine to deoxycytidine 5′-phosphate in the presence of a nucleoside 5′-triphosphate phosphate donor. The enzyme has been isolated and purified from many sources (Kessel, 1968; Momparler & Fischer, 1968; Durham & Ives, 1969, 1970; Kazai & Sugino, 1971; Coleman et al., 1975; Cheng et al., 1977; Meyers & Kreis, 1976; Hurley et al., 1983; Sarup & Fridland, 1987), and most investigators found that the same enzyme fraction also phosphorylated purine deoxyribonucleosides but with lower efficiency. The best studied enzyme, isolated from calf thymus, showed a broad substrate specificity, phosphorylating both pyrimidine and purine nu-

† This work was supported by grants from the Swedish Medical Research Council, the Swedish Cancer Society, and the Medical Faculty of the Karolinska Institute.

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0006-2960/88/0427-4258$01.50/0

cleosides and nucleoside analogues, using a variety of nucleoside triphosphates as phosphate donors (Momparler & Fischer, 1968; Durham & Ives, 1970; Ives & Durham, 1970; Kretinsky et al., 1976). A distal end product of the pathway, dCTP, is an inhibitor which probably serves as the physiological feedback regulator.

Studies with human somatic cell mutants resistant to deoxycytidine analogues support the conclusion that the same enzyme phosphorylates both purine and pyrimidine nucleosides (Verhoef et al., 1981; Hershfield et al., 1982) since mutant cells that lack dCyd kinase activity also show reduced capacity to phosphorylate deoxyadenosine and deoxyguanosine.

Despite this background information, considerable controversy exists regarding the substrate specificity of dCyd purified from various sources. For instance, cytoplasmic dCyd kinase from human leukemic cells was shown to be specific for deoxycytidine and arabinosylcytidine (Coleman, 1975; Cheng et al., 1977). Hurley et al. (1983) have found human placental dCyd kinase activity associated with deoxyadenosine or deoxyguanosine phosphorylating activities, but no dCyd kinase activity capable of using both purine substrates. Furthermore, an apparently homogeneous murine dCyd kinase unable to phosphorylate purine deoxyribonucleosides has been described (Meyers & Kreis, 1976, 1978a,b).

dCyd kinase plays a central role in DNA precursor biosynthesis in tissues with active nucleoside salvage pathways, such as thymus and spleen. This fact has recently been clarified by a series of studies on the mechanism of immunodeficiency, associated with lack of adenosine deaminase and purine nucleoside phosphorylase (Giblett et al., 1972, 1975; Carson et al., 1977; Cohen et al., 1978a,b, 1983; Martin & Gelfand, 1981). The underlying mechanism is thought to be accumulation of dATP and dGTP, respectively, both of which inhibit ribonucleotide reductase. This enzyme is responsible for the de novo synthesis of deoxyribonucleotides necessary for DNA synthesis. Accumulation of purine deoxyribonucleotides in both deficiencies is initiated by the action of nucleoside kinases. In these diseases, T-lymphocyte dysfunction is much more severe than B-lymphocyte dysfunction. One of the reasons for this difference is an increased level of active dCyd kinase in T lymphocytes as compared to B lymphocytes (Carson et al., 1977; Osborne, 1986). In addition, the existence of a T-lymphocyte-specific dCyd kinase has been reported (Yamada et al., 1985).

To clarify the role of dCyd kinase in relation to DNA precursor biosynthesis in various tissues and cells, we have purified the human enzyme, using leukemic spleen as starting material. A preliminary description of this work has been published (Bohman & Eriksson, 1986). Here we present the complete purification procedure for dCyd kinase, as well as basic properties of the apparently homogeneous enzyme.

Experimental Procedures

Materials

Tris-acryl DEAE and Ultrogel AcA-44 were purchased from LKB, Sweden, and hydroxylapatite (Hypatite C) was from Clarkson Chemical Co. Streptomycin sulfate was obtained from Sigma Chemical Co., and ultrapure ammonium sulfate was purchased from Merck.

[5-^{3}H]Deoxycytidine (28 Ci/mmol), [1′,2′-^{3}H]deoxyguanosine (26 Ci/mmol), and [1′,2′,2,8-^{3}H]deoxyadenosine (68 Ci/mmol) were purchased from the Radiochemical Centre, Amersham. Unlabeled nucleosides and nucleotides were obtained from Serva and Sigma Chemical Co. All other reagents were commercial preparations of the highest purity available.

Methods

Enzyme Assay. Deoxycytidine kinase was assayed with a radiochemical method which measures the formation of deoxyribonucleotides from labeled deoxyribonucleosides, using a modification of the assay method described by Ives and Wang (1978).

Deoxycytidine kinase in crude and partially purified extracts was assayed at 37 °C in a total volume of 250 μL, containing 40 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 8.0), 5 mM ATP, 5 mM $MgCl_2$, 0.6 mM deoxyuridine, 20 mM dithiothreitol, 15 mM sodium fluoride, 0.5 mM cytidine, and 10 μM [5-^{3}H]deoxycytidine (250 cpm/pmol). Conditions for the deoxycytidine kinase assay in pure extracts were 40 mM Tris-HCl (pH 8.0), 1 mM ATP, 1 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mg/mL bovine serum albumin, and [5-^{3}H]deoxycytidine at the concentrations indicated. When purine deoxyribonucleosides were used as substrates, the reaction mixture was the same as above except that it contained [1′,2′-^{3}H]deoxyguanosine or [1′,2′,2,8-^{3}H]-deoxyadenosine.

Assays were performed at 37 °C, and the enzyme concentration was adjusted to give no more than 25% conversion of substrate to product during 30-min incubation. Aliquots (50 μL) of reaction mixtures were spotted onto Whatman DE-81 filter paper disks after 1, 10, 20, and 30 min, respectively. The disks were immediately put into water and were then washed 3 times for 30 min in 3 L of 1.5 mM ammonium formate, with gentle stirring to keep them from settling into a heap and to remove unreacted substrate. The disks were subsequently washed once with water and once with ethanol. The washed disks were dried and then counted in a Packard Tri-Carb scintillation counter, using a toluene-based scintillation fluid containing 2,5-diphenyloxazole and 1,4-bis(5-phenyl-2-oxazolyl)benzene.

The amount of product formed was linear with time for more than 30 min provided that the 1-min value was subtracted from the later time points. One enzyme unit is defined as the amount of enzyme catalyzing the formation of 1 nmol of dCMP per minute under these assay conditions.

We found early during our investigation of dCyd kinase that the assay was not linear during the first minute, probably due to unspecific binding of nucleoside to the filters. However, if the 1-min value was subtracted from the later time points, there was a very good linear relationship between time and amount of product formed. The initial velocity was therefore measured from 1 min up to 19 or 29 min. Experimental values in the plots represent the average value between two time points. Lines between points have been drawn according to linear regression analysis.

Protein Determination. In fractions preceding the dTTP affinity chromatography step, protein concentration was determined by using Coomassie Brilliant Blue G-250 as described by Bradford (1976). After the dTTP–Sepharose step, protein concentration was determined according to Lowry as modified by Jovin et al. (1969) by using serum albumin as a standard. In the Ultrogel AcA-44 chromatogram, protein concentration was determined by measuring the absorbance at 280 nm.

Conductivity Measurements. All measurements were made at 4 °C with a CDM 3 Radiometer conductivity meter using a CDC 314 cell type.

Preparation of Thymidine Triphosphate Affinity Gel. Sepharose 4B was activated by cyanogen bromide as described by Kohn and Wilchek (1982), and a 3′,3′-diaminodipropylamine arm was attached and succinylated as described by Cuatrecasas (1970). To this linker was coupled a *p*-amino-

phenyl ester of dTTP (kindly provided by Dr. L. Thelander, Department of Physiological Chemistry, University of Umeå, Sweden). This nucleotide analogue was synthesized as described by Berglund and Eckstein (1972) with the modifications described by Knorre et al. (1976). The affinity column material contained approximately 0.5 μmol of dTTP/mL.

Source of Enzyme. Human adult leukemic spleens from patients suffering from various leukemic disorders were kindly supplied from the pathological department of the Karolinska Hospital. Normal spleens and human placentas were tested for dCyd kinase activity, and the enzyme activity was very low. Extracts from greatly enlarged spleens (approximately 2–2.5 kg) showed at least 10 times higher dCyd kinase activity as compared to the two other sources. The activity in leukemic spleen was similar to what was found in extract from human T-lymphoblast cells (CEM).

The spleens were chilled to 4 °C after splenectomy and frozen within 2 h and then stored at −70 °C.

Enzyme Purification. (A) Crude Extract Preparation. The minced tissue (approximately 0.7 kg) was suspended in an equal volume (w/v) of 50 mM potassium phosphate buffer (pH 7.5) containing 0.25 M sucrose, 5 mM dithiothreitol, 5 mM benzamidine, and 0.5 mM phenylmethanesulfonyl fluoride. Thereafter, the suspension was homogenized in a blender for five periods of 1 min at 4 °C, at which temperature all subsequent purification steps were performed. The homogenate was centrifuged at 9000 rpm in a Sorvall GSA rotor for 40 min.

(B) Streptomycin Sulfate Precipitation. To the supernatant from step A was added streptomycin sulfate to a final concentration of 0.7% and stirred for 30 min. The extract was centrifuged at 9000 rpm for 15 min.

(C) Ammonium Sulfate Fractionation. To the supernatant from step B was added crystalline ammonium sulfate to 20% saturation and stirred for 30 min. After centrifugation at 10 000 rpm for 15 min, more ammonium sulfate was added to the supernatant to give 60% saturation and stirred for 30 min. After centrifugation at 10 000 rpm for 15 min, the precipitate could either be stored at −70 °C or be suspended in an approximately equal volume (w/v) of 50 mM Tris-HCl buffer (pH 7.6) containing 20% glycerol, 5 mM $MgCl_2$, and 50 mM mercaptoethanol (buffer A). This protein solution was dialyzed overnight against 50 volumes of buffer A.

(D) Tris-acryl DEAE Chromatography. Aliquots (approximately 30%) of the dialyzed enzyme preparation from step C were adjusted to 2.7 mS by diluting with buffer A and applied to a Tris-acryl DEAE column (85 × 85 mm), equilibrated with the same buffer. The column was initially washed with 2 L of buffer A, and it was then eluted with a linear gradient of 0–0.33 M KCl in buffer A (total 8 L). Fraction volume was 150 mL. Deoxycytidine kinase activity was assayed in the various fractions (Figure 1), and those containing activity were pooled.

(E) Hydroxylapatite Chromatography. The pooled fractions (fractions 30–44 in Figure 1) containing the major enzyme peak from step D were applied to a hydroxylapatite column (90 × 40 mm) equilibrated with a 10 mM potassium phosphate buffer (pH 7.6) containing 5 mM $MgCl_2$, 20% glycerol, and 50 mM mercaptoethanol (buffer B). A linear gradient of 0.01–0.2 M potassium phosphate in 3 L of buffer B was applied to the column. Fraction volume was 25 mL. Deoxycytidine kinase activity eluted as a single peak with 0.075 M potassium phosphate (Figure 2). The pooled fractions (16–26) were dialyzed overnight against 12.5 volumes of buffer A.

Table I: Purification of Deoxycytidine Kinase from 0.7 kg of Leukemic Spleen

fraction	total vol (mL)	total act. (units)	total protein (mg)	sp act. (units/mg)
crude extract	365	260	14600	0.02
$(NH_4)_2SO_4$ (20–60%)	172	208	4990	0.04
Tris-acryl DEAE	690	76	445	0.17
hydroxylapatite	200	51	49	1.04
dTTP–Sepharose	4	35	0.3	115

(F) dTTP–Sepharose Chromatography. The dialyzed pool containing enzyme activity from step E was applied to a dTTP–Sepharose column (40 × 28 mm) equilibrated with buffer A. The column was washed with buffer A until no protein was detected in the eluate. At this time, the column was washed with 3 volumes of buffer A containing 0.07 M KCl. A linear gradient of 0–1.5 mM dTTP in 30 mL of buffer A, containing 0.07 M KCl, was then applied to elute the enzyme, and 1-mL fractions were collected. Deoxycytidine kinase activity eluted as a single peak (Figure 3) at approximately 0.8 mM dTTP. One polypeptide band which corresponded to the dCyd kinase activity was observed by sodium dodecyl sulfate (SDS) gel electrophoresis (Figure 3).

Analytical Techniques. (A) Gel Filtration on Ultrogel AcA-44. A fraction (4.25 mL) of the pooled enzyme after dTTP–Sepharose chromatography was ultradialyzed against 100 volumes of buffer A to 0.35 mL. An aliquot of the concentrated enzyme was applied to an Ultrogel AcA-44 (450 × 7.5 mm) column, equilibrated and eluted with 50 mM Tris-HCl (pH 7.6) buffer containing 5 mM $MgCl_2$, 5 mM dithiothreitol, 20% glycerol, and 0.1 M potassium chloride. The Ultrogel AcA-44 column was calibrated with transferrin, bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome *c*. The elution volume of the standard proteins was determined by following the absorbance at 280 nm.

(B) Glycerol Gradient Centrifugation. Concentrated enzyme (2.2 μg) after the dTTP–Sepharose step was applied to a 25–40% glycerol gradient, containing 50 mM Tris-HCl (pH 7.6), 0.1 M NaCl, 2 mM dithiothreitol, and 5 mM $MgCl_2$ in a total volume of 3.8 mL. Bovine serum albumin, ovalbumin, and chymotrypsinogen (approximately 200 μg of each) were added as references. Sedimentation was performed at 48 000 rpm in a Spinco Model L2-65B centrifuge in an SW 60 rotor for 43 h at 5 °C. Approximately 32 fractions of 0.1 mL were collected from one gradient by dripping from the bottom of the polyallomer Beckman tubes [size, $^7/_{16}$th in.; diameter, (2–3) × 8 in.], and dCyd kinase activity was determined. Aliquots of the fractions were analyzed by SDS gel electrophoresis to determine the peak positions of the marker proteins.

Results

Purification of Human dCyd Kinase. The high specific activity of dCyd kinase in extracts from human leukemic spleens enabled a large-scale purification of the enzyme as summarized in Table I. The apparently homogeneous enzyme had a specific activity of 110–150 nmol of dCMP produced min^{-1} (mg of protein)$^{-1}$. This is a 3–10-fold higher specific activity than reported for any other purified dCyd kinase.

In the initial purification scheme, a minor fraction (5–20%) of the dCyd kinase activity was eluted with low salt concentration during DEAE chromatography. However, upon rechromatography of this fraction, after further dialysis to reduce the conductivity (below 3 mS), the activity eluted at the same position as the major enzyme activity, i.e., with 0.2 M KCl (data not shown). Therefore, in the presently used purification

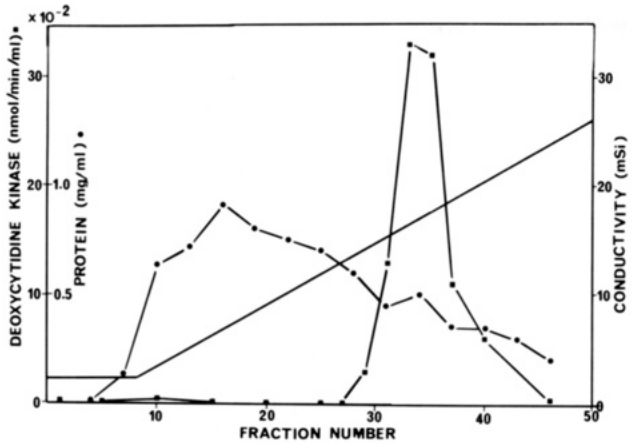

FIGURE 1: Ion-exchange chromatography of spleen cytosol proteins on Tris-acryl DEAE. The applied proteins were eluted with a linear KCl gradient, and the conductivity was determined (–). Enzyme activity (■) and protein concentration (●) were also determined in the fractions.

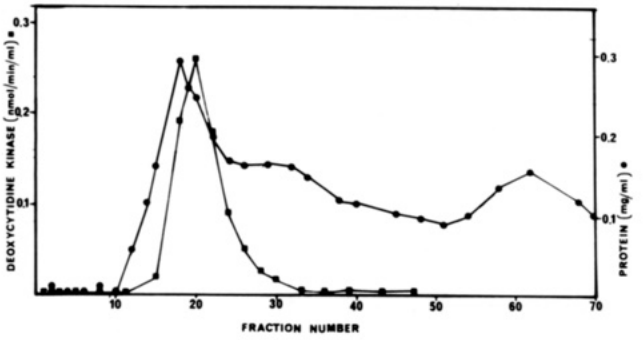

FIGURE 2: Hydroxylapatite chromatography of spleen deoxycytidine kinase. The pooled fractions, containing enzyme activity from a DEAE chromatogram, were adsorbed to a column of hydroxylapatite, and the column was eluted with a linear potassium phosphate gradient. Enzyme activity (■) and protein concentration (●) were determined in the fractions.

procedure, where the conductivity in the applied solution is below 3 mS, all of dCyd kinase activity elutes as one peak (Figure 1).

The most effective purification step was based on the high affinity of the enzyme for the phosphate donor dTTP (see below). Analysis, by SDS gel electrophoresis of the fractions containing dCyd kinase activity after dTTP–Sepharose chromatography, revealed one major band of molecular weight 30 000 (Figure 3A,B). Two other bands with apparent molecular masses of 60 and 67 kilodaltons (kDa) were observed, using the silver staining procedure, and they most likely represent contaminations in the mercaptoethanol as has been described earlier (Merril et al., 1984). Two-dimensionel gel electrophoresis (O'Farrel, 1975) of the pooled fractions after dTTP–Sepharose chromatography showed a double spot corresponding to dCyd kinase with isoelectric points of pH 5.5 and 5.7, respectively (data not shown). In a separate dTTP–Sepharose chromatogram, enzyme activity was measured, using (10 μM) deoxycytidine, (30 μM) deoxyguanosine (dGuo), or (30 μM) deoxyadenosine (dAdo) as substrate. All three activities followed the protein profile and showed the same relative ratio in all fractions. In summary, this purification procedure resulted in an apparently homogeneous preparation of dCyd kinase, capable of phosphorylating dGuo and dAdo in addition to dCyd. The overall yield was 10%.

Stability of the Enzyme and Absence of Contaminating Enzyme Activities. The final enzyme preparation after concentration and addition of bovine serum albumin to 0.5 mg/mL was stable in a Tris buffer containing $MgCl_2$, mercaptoethanol, and glycerol (buffer A) for at least 2 weeks when stored at 4 °C, and at –70 °C for at least 12 months. The pure enzyme was stable also in a buffer lacking reducing agents. In the absence of albumin, enzyme activity was lost upon repeated freezing and thawing.

Analysis by poly(ethylenimine) thin-layer chromatography of the products formed during the reaction showed that no detectable conversion (less than 1%) of the nucleoside monophosphates to di- or triphosphates occurred. No phosphatase activity was present, as judged by the fact that added deoxyribonucleoside triphosphates were completely stable during the standard reaction conditions.

Analytical Gel Filtration Chromatography and Glycerol Gradient Centrifugation. The enzyme activity eluted as a single peak on Ultrogel AcA-44 chromatography, and there was a direct correlation between the intensity of the polypeptide band of 30 kDa, observed with SDS gel electrophoresis, and the enzyme activity (data not shown). By empiric cor-

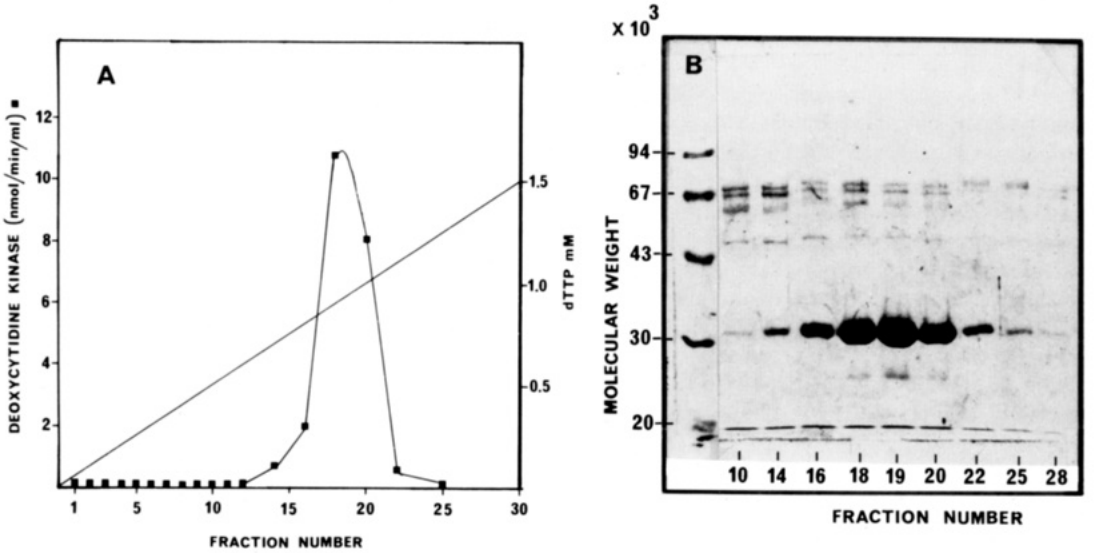

FIGURE 3: (A) Affinity chromatography of spleen deoxycytidine kinase on dTTP–Sepharose. The pooled fractions, containing the enzyme activity from a hydroxylapatite chromatogram, were adsorbed to a column of dTTP–Sepharose. Deoxycytidine kinase activity (■) was eluted with a linear dTTP gradient. (B) SDS gel electrophoresis of the fractions from dTTP–Sepharose chromatography. Aliquots of the fractions indicated were precipitated and analyzed by 10% SDS gel electrophoresis.

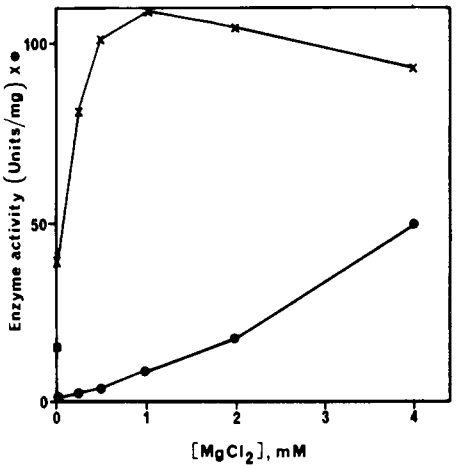

FIGURE 4: $MgCl_2$ concentration dependence curve for deoxycytidine kinase in the presence (●) and absence (×) of 2 mM EDTA. The substrate concentrations used were 2 μM dCyd and 2 mM ATP.

relation of the log of the molecular weight of the standard proteins with the reduced volume (V_e/V_0), a molecular weight of 60 000 could be estimated for dCyd kinase. The Stokes radius, calculated by the method of Ackers (1967), is 34 Å, and the free diffusion coefficient derived from the Stokes–Einstein equation (Siegel & Monty, 1966) is 6.3×10^{-7} cm^2 s^{-1}.

Analysis of pure dCyd kinase by glycerol gradient centrifugation demonstrated a single peak of activity. An $s_{20,w}$ of $(4.38 \pm 0.40) \times 10^{-13}$ s^{-1} was calculated according to the method of Martin and Ames (1961) from the position of enzyme activity and the peak positions of bovine serum albumin, ovalbumin, and chymotrypsinogen, respectively.

Using the parameters determined above and assuming a partial specific volume of 0.725, the molecular mass of human dCyd kinase is 61 ± 5 kDa, calculated according to the equation in the paper by Siegel and Monty (1966).

Requirements for the Enzyme Reaction. The enzyme showed a broad plateau of activity between pH 5.5 and 8.5 in acetate, Tris-HCl, phosphate, and Tris–glycine buffers of equal ionic strength. Phosphate buffers gave about 50% lower activity than Tris buffers at pH 7.5. Addition of dithiothreitol (2–10 mM) during the reaction gave a 30% stimulation.

Addition of $MgCl_2$, using 2 mM ATP as phosphate donor, gave maximal stimulation at 1–5 mM and inhibition at higher concentrations (Figure 4). Thus, the optimal ratio between Mg^{2+} and ATP is 0.5–2.5 for the pure enzyme. Due to the presence of $MgCl_2$ in the concentrated enzyme solution, we found no absolute dependence of Mg^{2+} unless ethylenediaminetetraacetic acid (EDTA) was added. The presence of chelator led to a complete inhibition of dCyd kinase activity, which was reversed by the addition of Mg^{2+} (Figure 4).

Kinetic Properties of the Enzyme. We have made a kinetic study with pure enzyme and the phosphate acceptors deoxycytidine, deoxyguanosine, deoxyadenosine, and the nucleoside analogue arabinosylcytosine (AraC). An ordinary hyperbolic saturation curve was found with 0.2–20 μM dCyd, which on a double-reciprocal plot could be fitted to a straight line with an apparent K_m of 1 μM (Table II). The other tested substrates gave similar plots, but the K_m values for dAdo and dGuo were 120 and 150 μM, respectively, and for AraC, it was 20 μM (Table II). However, the maximal velocity of the reaction was severalfold higher with the purines as compared with the pyrimidine substrates. Still, due to the large difference in K_m, the relative efficiency of dCyd as phosphate acceptor measured by the ratio between V_{max} and K_m is 27-fold higher compared to the purine substrates and 21-fold higher compared to the pyrimidine analogue (Table II).

Table II: Phosphate Acceptors for Human dCyd Kinase[a]

substrate	app K_m (M)	app V_{max} (nmol·min^{-1}·mg^{-1})	V_{max}/K_m ratio
dCyd	1.0×10^{-6}	145	145
AraC	2.0×10^{-5}	140	7
dGuo	1.5×10^{-4}	800	5
dAdo	1.2×10^{-4}	620	5

[a] Kinetic parameters were calculated by linear regression of Lineweaver–Burk plots, using 1 mM ATP·$MgCl_2$ as phosphate donor. The data represent the average of at least two separate experiments, and the values differed by less than 25%.

Table III: Competition between Various Phosphate Acceptors for Human dCyd Kinase[a]

substrate	inhibitor	app K_i (M)
dCyd	dGuo	2.8×10^{-3}
dCyd	dAdo	3.2×10^{-3}
dCyd	AraC	2.8×10^{-4}
dAdo	dCyd	2.5×10^{-7}
dAdo	dGuo	3.0×10^{-4}
dAdo	AraC	5.0×10^{-6}
dGuo	dCyd	1.0×10^{-7}
dGuo	dAdo	1.5×10^{-4}
dGuo	AraC	3.0×10^{-6}

[a] K_i values were determined from replots of the slopes of Lineweaver–Burk regression lines. Values are the average of three separate determinations, varying by less than ±25%.

Only very high concentrations of dGuo or dAdo were able to inhibit the phosphorylation of dCyd. Double-reciprocal plots showed that the inhibition was of a linear competitive type with an apparent K_i of 2.8 mM for dGuo and 3.2 mM for dAdo, respectively (Table III), using variable dCyd (0.2–10 μM) concentrations and constant ATP·$MgCl_2$ concentration (1 mM). AraC was a somewhat more efficient competitive inhibitor of the phosphorylation of dCyd, giving similar double-reciprocal curves and an apparent K_i value of 0.28 mM (Table III).

In contrast, the phosphorylation of dAdo by the human dCyd kinase was very strongly inhibited by the presence of dCyd. Double-reciprocal plots showed competitive inhibition with a K_i for dCyd of 0.25 μM, using variable dAdo concentrations (10–120 μM) and constant ATP·$MgCl_2$ concentration (1 mM) (Table III). Similar experiments were performed with AraC and dGuo as inhibitors of dAdo phosphorylation, demonstrating competitive inhibition but with higher K_i values (Table III).

Inhibition of the phosphorylation of dGuo by the presence of the other nucleoside substrates followed the same pattern as for the phosphorylation of dAdo. Thus, dCyd gave efficient competitive inhibition, while dAdo and AraC inhibited at higher concentrations (Table III).

Earlier studies of dCyd kinase have shown that a variety of nucleoside triphosphates can serve as phosphate donors for the enzyme. In this study, we have tested ATP and dTTP as substrates, using equimolar $MgCl_2$ concentration, which was found to be optimal, and three fixed concentrations of dCyd. Figure 5 shows a double-reciprocal plot comparing varying concentrations of ATP and dTTP and dCyd concentration fixed at 1, 2, and 10 μM, respectively. When ATP, at concentrations of 10 μM–1 mM, was used as a phosphate donor, the resulting data gave straight lines in double-reciprocal plots. The apparent K_m values for ATP differ slightly from 6 to 8 μM, using 1 or 10 μM dCyd, respectively. At ATP concentrations below 10 μM, nonlinear plots with transitions in the slopes were found, indicating lower apparent K_m values (2–3

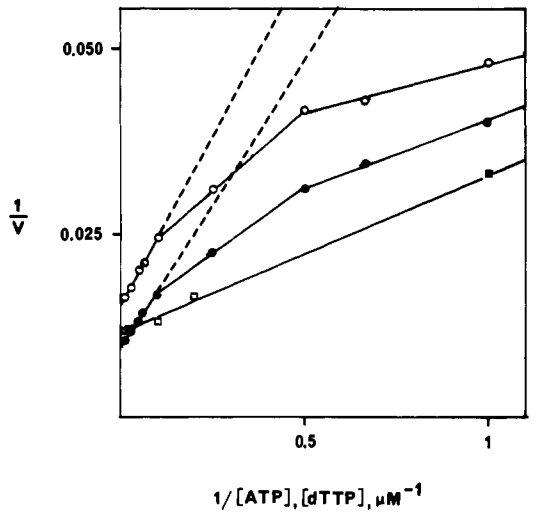

FIGURE 5: Effect of varying ATP·$MgCl_2$ and dTTP·$MgCl_2$ concentrations on the reaction rate with dCyd concentration fixed at 1, 2, and 10 μM, respectively. (□) 2 μM dCyd and varying concentrations of dTTP; (○) 1 μM dCyd and varying concentrations of ATP; (●) 10 μM dCyd and varying concentrations of ATP.

μM). Similar experiments with varying concentrations of ATP and fixed concentrations of dGuo and dAdo, at 150 μM, showed the same pattern with the experimental values on straight lines in double-reciprocal plots from 10 μM up to 1 mM ATP. The apparent K_m value was in both cases 14 μM, but below 10 μM ATP the slopes were also nonlinear with the purine substrates (data not shown).

When dTTP was used as a phosphate donor, a less complex pattern was found. Varying concentrations of dTTP from 1 μM to 1 mM and fixed concentration of dCyd at 2 μM resulted in experimental values on a straight line in the double-reciprocal plot, with an apparent K_m value of 2 μM (Figure 5).

The inhibition pattern of various nucleotides on the phosphorylation of dCyd (at 1 μM) was investigated, using 1 mM ATP·$MgCl_2$ as phosphate donor. As expected, dCTP was a very effective inhibitor, giving 50% inhibition of the activity at 2 μM, while the product dCMP, or dCDP, was much less effective (inhibiting 50% with 76 and 20 μM, respectively). The pyrimidine ribonucleotides CTP and UTP were only inhibitory at 100-fold higher concentrations than dCTP (280 and 200 μM, respectively).

The nature of dCTP inhibition was investigated, by using varying concentrations of dCyd (0.2–5 μM) and constant ATP·$MgCl_2$ concentration at 1 mM. A noncompetitive mixed type of inhibition was observed with an apparent K_{ii} of 3 μM and a K_{is} of 6 μM, using the slope and intercept of double-reciprocal plots for determination of the values. In contrast, an analysis of dCTP inhibition with constant dCyd concentration at 1 μM and varying ATP·$MgCl_2$ concentration (10–500 μM) indicated competition between these nucleotides with a low apparent K_i of 0.03 μM. A detailed product inhibition study with pure dCyd kinase is now in progress.

Discussion

The purification of dCyd kinase to apparent homogeneity has been obtained, using human leukemic spleen as starting material. This source was found to contain as much dCyd kinase activity as human T-lymphoblast (CEM) cell extracts. We found only one peak of dCyd kinase activity during DEAE chromatography (Figure 1), eluting with 0.2 M KCl. Yamada et al. (1985) have described the isolation and properties of a dCyd kinase specific for T lymphocytes that eluted with much lower salt during DEAE chromatography. We have used spleen from different types of leukemia, predominantly poorly differentiated B-cell lymphomas, but also extracts from CEM T-lymphoblast tissue culture cells. In no case did we observe significant amounts of different isoenzyme forms of dCyd kinase.

Pure dCyd kinase is remarkably stable, provided the protein concentration is kept high, and this stability of the enzyme is in contrast to what was found earlier with partially purified human dCyd kinase (Coleman et al., 1975; Cheng et al., 1977). The use of several protease inhibitors, high concentration of reducing agents and glycerol throughout the purification, may explain this fact. The Mg^{2+} requirement of the pure dCyd kinase was demonstrated by treatment with a chelator. Relatively low concentrations of Mg^{2+} gave maximal activity, and thus the enzyme accepts a wide range of Mg^{2+}·ATP concentrations for optimal activity.

The molecular weight and isoelectric point of the pure enzyme agree very well with previous studies of the partially purified calf thymus and human dCyd kinases. We show here for the first time that the active human enzyme is composed of two subunits of 30 kDa. Pure dCyd kinase phosphorylates deoxycytidine, deoxyguanosine, and deoxyadenosine, which demonstrates that the same enzyme is responsible for the phosphorylation of both purine and pyrimidine nucleosides. In this respect, our results confirm what was found for partially purified calf thymus enzyme (Durham & Ives, 1970; Ives & Wang, 1978) and in some studies on the human cytosolic enzyme (Hersfield et al., 1982; Osborne, 1986; Sarup & Fridland, 1987), but they disagree with several other studies on the human enzyme (Coleman et al., 1975; Cheng et al., 1977; Hurley et al., 1983).

During preparation of this paper, Sarup and Fridland (1987) reported the purification of dCyd kinase from human leukemic cells. They achieved a 2000-fold purification of the enzyme, with a specific activity of 45 nmol of dCMP formed per minute. Thus, although they could not determine the subunit composition of the enzyme, their data are in good agreement with what was found in this study.

The only other completely purified dCyd kinase, obtained from mouse sarcoma cells (Meyers & Kreis, 1976, 1978a,b), differs in several important aspects from what we found with the human enzyme. The murine dCyd kinase was composed of two subunits of molecular weights 38 000 and 39 000, respectively. It had a 10-fold higher K_m value and 3-fold lower V_{max} value compared to the human enzyme and could only phosphorylate dCyd and AraC. Therefore, apparently there are considerable species variations among mammalian cells for the structure and function of dCyd kinase.

A mitochondrial form of deoxycytidine kinase has been described by Cheng et al. (1977) that was capable of phosphorylating thymidine in addition to dCyd but with considerable higher K_m values. We have not found detectable amounts of a mitochondrial type of enzyme following this purification procedure.

The enzyme kinetic studies of the pure human dCyd kinase were designed to provide some basic characteristics of the reaction and specifically of test if there were indications of separate sites for purine and pyrimidine nucleosides. A dCyd kinase with separate interacting sites for the phosphorylation of dAdo and dCyd has been isolated and characterized from *Lactobacillus acidophilus* (Diebel et al., 1977a,b). However, the kinetic behavior of the *Lactobacillus* enzyme is different from what we observed with the human enzyme. We find clear-cut competition between all the nucleoside substrates tested, indicating a common nucleoside acceptor binding do-

main on the human dCyd kinase.

Our limited characterization of the phosphate donors of the enzyme was guided by the assumption that in vivo, ATP at approximately 1–2 mM concentration will most likely serve this function. At low concentrations of ATP, nonlinear plots were observed, and our results are in this respect similar to what was found earlier by Ives and Durham (1970), and this type of behavior of dCyd kinase has been interpreted to represent negative cooperative effects. In this study, dTTP as phosphate donator showed no deviation from linear plots and may thus bind to only one site (or form of the enzyme), but further studies are required to clarify this difference. Clearly, there are binding sites on the enzyme with high affinity for nucleoside triphosphates, a property which was used for the purification of the enzyme.

We found that dCTP was a very efficient inhibitor of the enzyme as compared to other pyrimidine nucleotides, including the product dCMP, which is in agreement with earlier studies. Competition between ATP and dCTP, but not between dCyd and dCTP, could also be demonstrated. Therefore, the inhibition of dCyd kinase by dCTP seems to be regulated by the ratio between dCTP and ATP, which probably also is the case in vivo.

We have observed a nonhyperbolic transition in the substrate saturation curve for pure dCyd kinase with concentrations of dCyd between 40 and 80 μM (unpublished result). This results in a bimodal character of the double-reciprocal plots for dCyd as phosphate acceptor, and similar results were reported both by Durham and Ives (1970) and by Sarup and Friedland (1987). Thus, there are indications of negative cooperative effects with both substrates of this enzyme. Furthermore, the large discrepancy between the apparent K_m value for a certain substrate and its corresponding K_i when used as an inhibitor also indicates the existence of allosteric effects.

Our present hypothesis to explain the kinetic properties of dCyd kinase postulates that the enzyme exists in two forms but with essentially the same catalytically active domain: one (more open) form capable of phosphorylating both purine and pyrimidine nucleosides and one (more closed) form, which is more selective, phosphorylating only pyrimidines. The transition between the two forms is governed by the substrates and/or nucleoside triphosphates binding to the substrate site or to effector site(s).

This type of model is inspired by the analogy with other very well-characterized kinase enzymes such as hexokinase and adenylate kinase (Anderson, 1979; Pai et al., 1977). These enzymes exist in two conformational states, as demonstrated by crystallographic studies, and the transition between one form and the other is induced by the binding of substrates. A bilobal structure has been found in these and other phosphoryl transfer enzymes with a deep cleft between the two lobes. A dramatic conformational change occurs upon binding of substrates to the bottom of the cleft, which leads to the closing of the cleft.

Further structural, kinetic, and direct binding studies are needed to clarify the relevance of this analogy with regard to human dCyd kinase.

Registry No. dCyd, 951-77-9; AraC, 147-94-4; dGuo, 961-07-9; dAdo, 958-09-8; dCyd kinase, 9039-45-6.

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Purification and Physicochemical Characterization of a Human Placental Acid Phosphatase Possessing Phosphotyrosyl Protein Phosphatase Activity†

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Received May 20, 1987; Revised Manuscript Received February 18, 1988

ABSTRACT: A 17-kilodalton (kDa) human placental acid phosphatase was purified 21 400-fold to homogeneity. The enzyme has an isoelectric point of pH 7.2 and a specific activity of 106 μmol min^{-1} mg^{-1} using *p*-nitrophenyl phosphate as a substrate at pH 5 and 37 °C. This placental acid phosphatase showed activity toward phosphotyrosine and toward phosphotyrosyl proteins. The pH optima of the enzyme with phosphotyrosine and with phosphotyrosyl band 3 (from human red cells) were between pH 5 and 6 and pH 5 and 7, respectively. The K_m for phosphotyrosine was 1.6 mM at pH 5 and 37 °C. Phosphotyrosine phosphatase activity was not inhibited by tartrate or fluoride, but vanadate, molybdate, and zinc ions acted as strong inhibitors. Enzyme activity was also inhibited by DNA, but RNA was not inhibitory. It is a hydrophobic nonglycoprotein containing approximately 20% hydrophobic amino acids. The average hydrophobicity was calculated to be 903 cal/mol. The absorption coefficient at 280 nm, $E^{1\%}_{1cm}$, was determined to be 5.7. The optical ellipticity of the enzyme at 222 nm was −5200 deg cm^2 dmol^{-1}, which would correspond to a low helical content. Free sulfhydryl and histidine residues were necessary for the enzyme activity. The enzyme contained four reactive sulfhydryl groups. Chemical modification of the sulfhydryls with iodoacetate resulted in unfolding of the protein molecule as detected by fluorescence emission spectroscopy. Antisera against both the native and the denatured protein were able to immunoprecipitate the native enzyme. However, upon denaturation, the acid phosphatase lost about 70% of the antigenic determinants. Both antisera cross-reacted with a single 17-kDa polypeptide on immunoblotting.

Phosphorylation and dephosphorylation of proteins at serine residues represent one important mechanism for the regulation of enzymes in eukaryotes (Cohen, 1982; Ingebristen & Cohen, 1983). The phosphorylation of tyrosyl residues, which is thought to be more rare, has been suggested to be involved in cell proliferation and differentiation (Hunter & Cooper, 1985). In general, the phosphorylation state of any such protein, and therefore the physiological processes it controls, may be the result of a balance between competing protein kinase and protein phosphatase activities (Frank & Sartorelli, 1986). The physiological significance of tyrosine kinase in the regulation of cell proliferation and in transformation has been very well documented (Hunter & Sefton, 1980; Cooper et al., 1982). In contrast, very little is known about the role of phosphotyrosyl phosphatases. Recently, Klarlund described exciting experiments in which vanadate, an inhibitor of certain phosphatases, was able to transform NRK-1 cells (Klarlund, 1985). Transformation of the cells by vanadate was accompanied by an increase in the phosphotyrosyl content. This increase in the phosphotyrosyl content of the cellular proteins may be due to an increase in the tyrosine kinase level, but it could also result from an inhibition of phosphotyrosyl phosphatases by vanadate. There is evidence that pp60$^{v\text{-}src}$ kinase shows increased activity upon vanadate treatment (Brown & Gordon, 1984). At the same time, vanadate and related early transition-metal oxoanions possess broad potential as transition-state analogues of phospho transfer reactions in general (Van Etten et al., 1974), and of acid phosphatases in particular (Van Etten et al., 1974; Van Etten, 1982). More recently, vanadate has been shown to act as an inhibitor of phosphotyrosyl phosphatases (Swarup et al., 1984; Okada et al., 1986). Thus, elevated levels of phosphotyrosine in cellular proteins may be the result of decreased phosphatase activities as well as from increased tyrosine kinase activities.

Several acid phosphatases of differing molecular weights have been characterized in mammalian tissues (Heinrikson, 1969; Ostrowski et al., 1976; Lawrence & Van Etten, 1981), and some of them show phosphotyrosyl phosphatase activity (Okada et al., 1986; Li et al., 1984; Lin & Clinton, 1986; Lau et al., 1985; Chernoff & Li, 1985; Shriner & Brautigan, 1984; Apostol et al., 1985). High molecular weight (lysosomal) and low molecular weight acid phosphatases from human liver have been purified and characterized (Saini & Van Etten, 1978; Taga & Van Etten, 1982). It was observed that these enzymes efficiently hydrolyze phenyl phosphate, although their activity against phosphotyrosyl-containing proteins was not specifically tested. From earlier work (DiPietro & Zengerle, 1967; Rehkop & Van Etten, 1975), it was clear that human liver and placenta contain at least three different types of acid

†This research was supported by USDHHS Research Grant GM 27003 from the National Institute of General Medical Sciences.
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0006-2960/88/0427-4265$01.50/0